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**14. ABSTRACT**

Background: The majority of patients with advanced breast cancer develop bone metastases, which are incurable. Recently, tumor-secreted factors have shown promise as targets for the treatment of bone metastasis. Adrenomedullin (AM) is a breast cancer-secreted peptide that is pro-proliferative, anti-apoptotic, pro-angiogenic, and stimulates new bone formation. AM overexpression increases bone metastases while AM knockdown decreases bone metastases in mouse models of prostate and lung cancer respectively. Objective/Hypothesis: The objective of this project is to validate AM as an important target for the treatment of breast cancer bone metastasis. I hypothesize that AM expression increases bone metastases and resistance to chemotherapy. Specific Aims: (1) To determine if AM expression by breast cancer cells increases bone lesion formation in bone metastasis mouse models. (2) To determine the role of AM in breast cancer cells. Key Research Accomplishments: (1) A human AM expression vector has been produced and is being used to make stable MCF-7 AM-overexpressing cells. (2) Stable AM shRNA knockdown MDA-MB-231 breast cancer cell clones have been produced and will be ready for mouse heart injection in June. (3) Current evidence does not indicate that AM is regulated by RhoGDI2 in breast cancer cells. Relevance: Currently no treatments improve overall survival for breast cancer bone metastasis patients. Studying AM may lead to the development of an adjuvant therapy to improve treatment.

**15. SUBJECT TERMS**
bone metastasis, adrenomedullin, tumor-secreted factors

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**Introduction:**

The majority of patients who develop advanced breast cancer develop bone metastases, which are incurable (Siclari et al, 2007). Bone metastasis patients suffer from extreme bone pain, skeletal fractures, nerve compression, and hypercalcemia (Siclari et al, 2007). Current treatment, the use of antiresorptive bisphosphonates, reduces bone pain and skeletal fractures but does not improve overall survival (Siclari et al, 2007). The incurability of the disease is produced by a 'vicious cycle' that develops between the tumor cell and the bone microenvironment (Siclari et al, 2007). Once the tumor cell has entered bone, the tumor cell secretes factors that act on bone cells and other surrounding cells, causing them in turn, to secrete factors back onto the tumor cells (Siclari et al, 2007). Inhibiting tumor-secreted factors has led to decreased bone metastases in mice (Siclari et al, 2007). Currently, inhibitors of two tumor-secreted factors are under clinical trials for bone metastasis treatment (Siclari et al, 2007). My project is studying the tumor-secreted factor adrenomedullin (AM) and its role in breast cancer bone metastasis. AM is a 52 amino acid peptide that is pro-proproliferative, anti-apoptotic, pro-angiogenic, and induces new bone formation (Zudaire et al, 2003, Cornish et al, 2003). Overexpressing AM increased bone metastasis formation while decreasing AM decreased bone metastasis formation in prostate and lung cancer mouse models respectively [unpublished data]. My hypothesis is that AM is a causal factor in breast cancer bone metastasis that increases lesion formation and chemoresistance. To test this hypothesis, I plan to look at the effect of stable overexpression and knockdown of AM in breast cancer cells on bone lesion formation, cell proliferation, chemoresistance, and cell migration and invasion.

**Body:**

**Task 1: Training in how to produce AM-overexpressing stable clones. Create and test stable AM-overexpressing MCF-7 cell lines for mouse study.**

The hAM gene was cloned into the pIRESneo3 expression vector, a bicistronic expression vector. The emerald green fluorescent protein gene was also cloned into this vector as a negative control. This expression vector was chosen to try to increase the number of clones that express both the antibiotic resistance gene and the hAM gene. A problem often encountered in this laboratory is that few of the single cell clones produced express the gene of interest. Using this vector allows both the AM gene and the antibiotic resistance gene to be expressed from the same promoter which should increase the number of cells that express both the antibiotic resistance gene and AM, hopefully increasing the number of AM-overexpressing single cell clones. Both the pIRES-hAM and emGFP expression vectors were tested. A 9444 fold increase in hAM was detected after 48 hr transient transfection of MCF-7 cells with pIRES-hAM using RealTime PCR (Figure 1) and green fluorescence was detected in MDA-MB-231 cells transfected with pIRES-emGFP using a fluorescent microscope (Figure 2).

MCF-7 (low AM-expressing) breast cancer cells have been transfected with pIRESneo3-hAM and emGFP and placed under selection and I am currently waiting for the stable pool to grow out. Once the stable pools grow out, single cell clones will be grown out. After the clones have been tested for stability, they will be placed into the mouse model and I will determine if increasing AM increases bone lesion formation and chemoresistance (Tasks 2, 3, and 7).

**Task 4: Training in the use of siRNA to produce stable AM-knockdown clones. Create and test stable AM knockdown MDA-MB-231 cells for mouse study using siRNA.**

Five short hairpin RNA (shRNA) expression vectors targeting hAM and a corresponding scrambled version of these sequences (negative control) were designed using the Ambion silencer express kit. The shRNA expression vectors were tested for their ability to knockdown human AM using a cotransfection method. Human AM was cotransfected along with the shRNA into rodent cells (CHO) that do not express hAM to compare the ability of the shRNAs to decrease hAM mRNA levels. Human AM mRNA levels were assessed by gel analysis of PCR products (Figure 3). The shRNA AM735 was chosen to produce a stable cell line because it produced the largest AM knockdown while its corresponding scrambled version did not affect AM mRNA levels. Stable AM735 and 735SCR pools were produced in MDA-231 cells (high AM expressing breast cancer cells) by transfecting the cells with a range of concentrations of shRNAs and using neomycin selection. The pool that grew up and was transfected with the least amount of shRNA DNA was used to make single cell clones (titration control for shRNA). The pools selected had a 19-fold decrease in AM between the AM735 shRNA pool and the SCR shRNA negative control pool (Figure 4). Producing single cell clones turned out to be a difficult task. After plating the cells using a limited dilution method, the cells repeatedly died. After reducing the neomycin concentration by about ten fold, I was finally able to produce single cells clones. Using real-time PCR, I determined relative AM mRNA levels of the clones and determined that the average difference in AM of the clones was about a nine-fold decrease in AM in the knockdown clones (Figure 5). The

clones were than cultured without selection to test the stability of the knockdown. The knockdown clones maintained their knockdown after thirty days of culture without neomycin treatment (Figure 5). Sixty day culture is currently underway. As soon as the clones pass the sixty day stability test, two knockdown clones, two control clones, and the parental cell line will be injected into mice to determine if there are any differences in bone lesion formation when AM is knocked down (Tasks 5 and 6). Experiment is tentatively planned for the beginning of June.

**Task 8: Test the gene changes induced by AM treatment of breast cancer cells using Q-PCR.**

The initial plan for this task was to determine if gene changes induced by AM in primary osteoblasts also occurred in breast cancer cells. However, validating gene changes found from a microarray of 24 hr AM-treated calvariae in primary osteoblasts turned out to be problematic. Of the 11 genes tested from the microarray, only 1 gene (Timp1) turned out to be statistically significantly changed in primary osteoblasts after AM treatment and this gene change was only slight and may not be physiologically relevant. Therefore, I concluded that using the microarray was not an effective method to determine gene changes induced by AM in primary osteoblasts and therefore not an effective method to determine gene changes in breast cancer cells.

Alternatively, I've been looking to see if there are gene changes in genes known to be important in bone metastasis in my MDA-MB-231 AM knockdown clones (Figure 6). There was a statistically significant decrease in both IL-11 and ET-1 in the AM knockdown clones compared to the control clones. AM has not been reported to regulate IL-11 however, AM has been reported to regulate ET-1 expression in hypoxic conditions. Whether or not these differences are a clonal variation, shRNA artifact, or an actual effect of AM knockdown has not been determined yet. There was no significant difference in Cyr61, PTHrP, CTGF, IL-8, and IL-6 between the knockdown and control clones.

**Task 9: Test role of Brca1 and Rho signaling in the regulation of AM expression in breast cancer cells.**

Using RealTime PCR, I confirmed an increase in the Rho inhibitor, RhoGDI2, and a decrease in AM mRNA levels in T47D COBRA2a knockdown cells compared to control T47D shEGFP cells (Figure 7). However, inhibiting a downstream effector of Rho (ROCK) in T47D shEGFP using the ROCK inhibitor Y-27632 had no effect on AM mRNA levels, suggesting that this downstream Rho effector (ROCK) is not involved in regulating AM expression (Figure 8). To further test if RhoGDI2 is involved in regulating AM mRNA transcription, I transiently transfected MCF-7 and MDA-MB-231 cells with a hRhoGDI2 expression vector. Overexpression of RhoGDI2 in MCF-7 cells did not significantly change AM mRNA levels determined using RealTime PCR (Figure 9). Overexpression of RhoGDI2 in MDA-MB-231 cells did not significantly change AM mRNA levels or AM promoter activity determined using RealTime PCR and an AM promoter luciferase reporter respectively (Figure 10). These studies suggest that my hypothesis is incorrect and AM is not regulated by RhoGDI2 in breast cancer cells.

**Task 10: Write papers and defend thesis project**

I have published a review entitled “Molecular interactions between breast cancer cells and the bone microenvironment drive skeletal metastases” in the Cancer and Metastasis Reviews Journal.

## Summary of Specific Aims and Completed Tasks:

**Specific Aim 1**: **To determine the effects of tumor AM on bone**

**Specific Aim 1.1: To determine the effects of increasing tumor-expressed AM in MCF7 cells on bone metastasis**

**Task 1:** (Specific Aim 1.1) Training in how to produce AM-overexpressing stable clones.
Create and test stable AM-overexpressing MCF7 cell lines for mouse study.
**Task in progress**

**Task 2:** (Specific Aim 1.1) Training in how to produce mouse models of bone metastasis and training in how to analyze mouse model results.
Create mouse models of breast cancer bone metastasis using stable AM-overexpressing MCF7 cells and control MCF7 cells.
**Task will be completed after Task 1 is complete.**

**Task 3:** (Specific Aim 1.1) Analyze data from mouse experiment for Specific Aim 1.1 using x-rays, bone histology, and histomorphometry.
Completion of this task will reveal whether overexpression of AM in MCF7 cells increases bone lesion formation in mice.
**Task will be completed after Task 1 is complete.**

**Specific Aim 1.2: To determine the effects of decreasing tumor expression of AM in MDA-MB-231 cells on bone metastasis**

**Task 4:** (Specific Aim 1.2) Training in the use of siRNA to produce stable AM-knockdown clones.
Create and test stable AM knockdown MDA-MB-231 cells for mouse study using siRNA.
**Task complete**

**Task 5:** (Specific Aim 1.2) Create mouse models of breast cancer bone metastasis using stable AM knockdown MDA-MB-231 clones and control MDA-MB-231 breast cancer cells.
**Task planned for beginning of June**

**Task 6:** (Specific Aim 1.2) Analyze data from mouse experiment for Specific Aim 1.2 using x-rays, bone histology, and histomorphometry.

Completion of this task will reveal whether decreasing the expression of AM decreases bone lesion formation in mice.
**Task will be completed in the next couple of months.**

**Specific Aim 2**: **To determine the role of AM in Breast Cancer cells**
**Specific Aim 2.1: To determine if AM increases resistance of tumor cells to chemotherapy**

**Task 7:** (Specific Aim 2.1) Test the effects of AM-overexpression in breast cancer cells on sensitivity to taxol using MTT assays.
Completion of this task will reveal whether AM promotes chemoresistance to taxol.
**Task will be completed after Task 1 is completed.**

**Specific Aim 2.2: To determine if genes regulated by AM in bone cells are also regulated similarly in breast cancer cells**

**Task 8:** (Specific Aim 2.2) Test the gene changes induced by AM treatment of breast cancer cells using Q-PCR
Completion of this task will reveal the mechanism of AM action in breast cancer. This task may also reveal additional targets for the treatment of breast cancer.
**Task underway**

**Specific Aim 2.3: To determine the regulation of AM expression in breast cancer cells by BRCA1 and whether this regulation is via Rho signaling and the metastasis suppressor Rho GDI2, a negative regulator of Rho kinase signaling**

**Task 9:** (Specific Aim 2.3) Test role of Brca1 and Rho signaling in the regulation of AM expression in breast cancer cells using siRNA, transient transfections, and Q-PCR.
Completion of this task will reveal whether or not Rho signaling regulates AM expression in Breast Cancer cells and will indicate a possible mechanism to decrease AM levels in Breast Cancer.
**Task complete. No evidence that RhoGDI2 regulates AM. Abandon task.**

**Task 10**: Write papers and defend thesis project
Completion of this task will result in the publication of at least one paper in a well-respected journal and the achievement of a Ph.D in biochemistry and molecular genetics.
**Task in progress**
**Review article was published in December.**

**Key Research Accomplishments:**

- Produced stable AM knockdown breast cancer cell clones that will be used to determine if decreasing AM in breast cancer cells decreases bone lesion formation in vivo.
- Produced a human AM expression vector that will be used to produce stable AM-overexpressing breast cancer cell clones that will be used to determine if increasing AM increases bone lesion formation in vivo.
- Found no evidence supporting hypothesis that AM is regulated by RhoGDI2 in breast cancer cells.
- Published a review article about breast cancer bone metastasis.

**Reportable Outcomes:**
12/2006 and 5/2007: Abstract/Poster Presentation at the VI International Meeting on Cancer Induced Bone Disease and the University of Virginia Department of Medicine Research Day
Title: Development of Small Molecule Adrenomedullin Antagonists for Treatment of Bone Metastases
12/2006: Review article published in Cancer and Metastasis Reviews
Title: Molecular interactions between breast cancer cells and the bone microenvironment drive skeletal metastases

**Conclusion:**

Since the current treatment for breast cancer bone metastases does not cure the disease, new treatments need to be developed. I hypothesize that the breast cancer-secreted peptide adrenomedullin (AM) is a causal factor in breast cancer bone metastasis and inhibiting AM may decrease breast cancer bone metastases. My experiments are set up to determine the role of AM in breast cancer bone metastasis by looking at the effects of both decreasing and increasing AM on breast cancer bone metastasis formation. To look at the effects of decreasing AM, I produced stable AM shRNA and control MDA-MB-231 breast cancer cell clones that will be used in the intracardiac injection mouse model to determine if decreasing AM expression decreases bone lesion formation. I am also working on producing stable MCF-7 AM-overexpressing breast cancer cells to determine if increasing AM expression increases bone lesion formation. The stable cell clones will also be used to determine in vitro if changing AM expression levels changes breast cancer sensitivity to chemotherapy, cell proliferation, and invasion. I also found no evidence to support the hypothesis that AM is regulated by RhoGDI2 in breast cancer cells, concluding aim 2.3.

If AM is a causal factor in breast cancer bone metastasis and leads to increased bone lesion formation, AM inhibitors may be a new adjuvant therapy for breast cancer bone metastasis treatment. Small molecule AM inhibitors have already been developed (Martinez et al, 2004). Small molecule AM inhibitors may therefore improve breast cancer bone metastasis treatment, reducing the pain and suffering associated with the disease.

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**Appendices:**

Abstract: Development of Small Molecule Adrenomedullin Antagonists for Treatment of Bone Metastases

Siclari VA, Mohammad KS, Martinez A, Gineste C, Geysen HM, Guise TA, Chirgwin JM

Adrenomedullin (AM) is a 52 amino acid peptide of the calcitonin/CGRP gene family. AM is secreted by cancers such as breast, lung, and prostate, where it can stimulate angiogenesis and autocrine signaling. AM also dose-dependently stimulates osteoblast proliferation and new bone formation at picomolar concentrations, by binding to the calcitonin-receptor-like receptor plus RAMP2 or 3 and stimulating adenyl cyclase. However, the mechanisms by which AM induces new bone formation are incompletely understood. We previously reported increased and decreased bone metastases due to AM overexpression and siRNA knockdown respectively in prostate and lung cancer models. These observations identify AM as a significant target for therapeutic intervention in bone metastasis.

Small molecules have been identified that function as agonists or antagonists of the action of AM to increase cAMP. They bind to the AM ligand rather than the receptor. One of these antagonists, NCI 16311, binds with Kd = 8nM to AM without altering receptor binding affinity. 100nM 16311 was added to cultures of neonatal mouse calvariae. The increases in new bone and osteoblast number caused by 1nM AM were completely blocked by 16311, without cellular toxicity or blockade of new bone formation due to IGF1 receptor activation. However, 20nmol/kg 16311 iv dramatically increased blood pressure in rats. Two additional antagonists were tested in the same assays. NCI 28086 was ineffective, but NCI 37133 was as effective as NCI 16311 at blocking AM-induced new bone formation, while it did not increase blood pressure in rats. Thus it may be possible to develop effective bone-selective antagonists of tumor-secreted AM. NCI compounds 16311 and 37133 are aromatic carboxylic acid derivatives that act extracellularly. Further development of these compounds into higher affinity, second-generation derivatives should be possible. Small molecule AM antagonists may lead to improved treatment for bone metastasis.

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# Molecular interactions between breast cancer cells and the bone microenvironment drive skeletal metastases

**V.A. Siclari · T.A. Guise · J.M. Chirgwin**



**Abstract** Breast cancer cells preferentially spread to bone. Bone metastases are currently incurable and therefore better treatments need to be developed. Metastasis is an inefficient, multi-step process. Specific aspects of both breast cancer cells and the bone microenvironment contribute to the development of bone metastases. Breast cancers express chemokine receptors, integrins, cadherins, and bone-resorbing and bone-forming factors that contribute to the successful and preferential spread of tumor to bone. Bone is rich in growth factors and cell types that make it a hospitable environment for breast cancer growth. Once breast cancer cells enter the bone, a highly complex vicious cycle develops, in which breast cancer cells secrete factors that act on bone cells and other cells within the bone (stem cells, T cells, platelets, adipocytes, fibroblasts, and endothelial cells), causing them to secrete factors that act on adjacent cancer cells. The steps in the metastatic cascade and the vicious cycle within bone offer unique targets for adjuvant treatments to treat and cure bone metastases.

**Keywords** Breast cancer · Bone metastasis · Bone microenvironment

## 1 Introduction

Breast cancer is one of several cancers including lung and prostate cancer that preferentially spreads to bone [1]. One out of every eight women will develop breast cancer during her lifetime (http://www.cancer.org/cancerinfo). Of those women who progress to an advanced stage of disease, over 80% will develop bone metastases [2]. Cancer cells enter bone through nutrient arteries and typically form metastases in the axial skeleton. In particular, they form in the vertebrae, pelvis, the proximal ends of the long bones, and the skull [3, 4]. Within bone, the breast cancer cells produce osteolytic (bone destructive) lesions most commonly, but also osteoblastic (bone formation), or mixed lytic and blastic bone lesions [2]. Once breast cancer has metastasized to bone, the cancer is incurable and patients suffer from extreme bone pain, skeletal fractures, hypercalcemia, and nerve compression [2]. Median survival for patients with bone metastases from time of diagnosis is about 2 years [2]. Current treatment with bisphosphonate antiresorptive drugs is palliative and does not improve overall survival [2]. Understanding the bone microenvironment, and why breast cancer cells preferentially spread to bone, should reveal new targets for the improved treatment of breast cancer bone metastasis.

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## 2 Breast cancer preferentially spreads to bone

Over a hundred years ago, Stephen Paget first noted the nonrandom spread of different types of cancer to distinct organs within the body unexplained by mere blood flow [5]. In an autopsy study of 735 women who died of breast cancer, Paget found that the two most common sites of metastasis were the ovaries and bone [3, 6]. Paget proposed an explanation for why cancer cells only form metastases in particular areas of the body: the seed and soil hypothesis, which states: "when a plant goes to seed, its seeds are carried in all directions; but they can only grow if they fall on congenial soil [6]." The 'seed' is the cancer cell, which after circulating through the blood stream, can only 'grow'

or form metastases in particular compatible areas of the body, 'the congenial soil' [6]. Not all cancers can grow on all 'soil' [5]. Both the seed and the soil contribute to the ability of the cancer to form site-selective metastases [5].

In the case of breast cancer, the bone provides a very 'congenial soil' in which the 'seed' can grow. More recent studies have confirmed bone as a common site of metastasis for breast cancer [3]. Three independent autopsy studies in 1948, 1979, and 1992 found that 62–71% of patients who died from breast cancer had bone metastases [7–9]. Coleman and Rubens in 1987 identified bone as the most common site of first distant relapse for breast cancer [10]. The molecular basis for the preferential metastasis to bone of breast cancers is the focus of this review. The characteristics of the breast cancer seed and the bone soil and their interactions will be reviewed and understudied areas highlighted.

## 3 The road to the bone: An inefficient multistep process

To successfully metastasize, a cancer must take a series of steps [5]. Cancer cells must (1) detach and extravasate from the primary tumor, (2) invade through extracellular matrix and endothelium into the blood stream, (3) survive the turbulent flow in the blood stream, (4) arrest at a distant site by adhesion to a specific endothelium, (5) extravasate across the endothelium and migrate through further extracellular matrix, and (6) finally halt and grow at a distant site [5]. The process is very inefficient and could fail at any step [5]. It is unknown which step is rate-limiting *in vivo*, but all steps are targets for potential *preventitive* treatments. We focus on step 6 and present understanding of *established* bone metastases and how these may be treated.

A primary tumor contains a heterogeneous population of cells with varying metastatic ability, some of which express pro-metastatic genes that enable the cells to spread to the bone [5, 11]. Once there, the bone microenvironment induces phenotypic changes in the cells [4]. The bone microenvironment alters gene expression by the breast cancer cells [4, 12]. In particular, the cancer cells increase their production of cytokines and other active factors that act on cells in bone, making the bone microenvironment more favorable for cancer colonization [4, 12]. Thus, the specific molecular interactions between the breast cancer cell and the bone microenvironment drive the formation of bone metastases.

Several characteristics of the breast cancer cell allow breast cancer cells to preferentially reach bone [2]. The chemokine receptors, integrins, matrix metalloproteinases, and several tumor-secreted factors aid breast cancer cells in the difficult steps (1–5, above) leading to bone [2].

## 4 Chemotaxis: Homing to bone

The CXC chemokine receptor-4 (CXCR4) is a G-protein coupled receptor that can activate breast cancer cell chemotaxis, tumor cell migration and proliferation, and angiogenesis [13]. Vascular endothelial growth factor (VEGF) increases CXCR4 expression [13]. CXCR4 expression in breast cancer cells also may be regulated by the hypoxia-induced Hif-1α pathway [13, 14]. Increased expression of CXCR4 on breast cancer cells has been associated with breast cancer progression [15].

CXCR4 is the receptor for stromal cell-derived factor-1 (SDF-1, also called CXCL12) [13]. The ligand is abundantly secreted by bone and other common sites of breast cancer metastasis [16]. An anti-CXCR4 antibody reduced chemotaxis of breast cancer cells toward bone marrow conditioned media [17].

Kang et al., identified CXCR4 as one of a set of five genes together responsible for breast cancer metastasis to bone [11]. Overexpression of CXCR4 in MDA-MB-231 breast cancer cells significantly increased bone metastases formation in nude mice [11]. Inhibition of CXCR4 using anti-CXCR4 antibodies reduced breast cancer metastases to the lung and lymph node after tail vein or mammary fat pad injection of the same cell line [17]; it also reduced bone metastases in a prostate cancer model [18]. Synthetic polypeptides and siRNAs against CXCR4 reduce lung metastases in mice receiving MDA-MB-231 cells by tail vein injection [19, 20]. Small molecule antagonists of CXCR4 have been developed to inhibit cellular entry of HIV [21]. They may be useful in reducing breast cancer metastasis to bone [2].

## 5 Cell adhesion: Integrin receptor αvβ3 and cadherins

Breast cancer cells need to adhere to bone to metastasize successfully to the skeleton [22]. Integrins are a family of transmembrane receptors composed of alpha and beta subunits, involved in cell–cell and cell–matrix adhesion [22]. Integrin alphavbeta3 (αvβ3, the vitronectin receptor) provides breast cancer and osteoclast adhesion to bone matrix [22]. It binds RGD (arginine–glycine–aspartic acid) peptide sequences present in several bone matrix proteins including osteopontin, vitronectin, and bone sialoprotein [22, 23].

Breast cancer cells metastatic to bone express high levels of αvβ3 [24]. Overexpression of a constitutively active αvβ3 increased lung metastasis formation by MDA-MB-435 cells injected into the tail vein of mice [25]. Expression of αvβ3 in the mammary carcinoma cell line 66cl4 caused spontaneous bone metastases in an orthotopic model of lung metastases [26]. S247, a small molecule antagonist of

αvβ3, reduced bone metastases by MDA-MB-435 cells [22]. Small molecule αvβ3 inhibitors could reduce bone metastases in breast cancer patients. S247 was only effective in prevention and not in a treatment model [22]. New specific small molecule αvβ3 inhibitors are currently being developed and could be used in the prevention of bone metastases [23]. In fact, the integrin antagonist Cilengitide is currently in phase II clinical trials in men with nonmetastatic androgen-independent and metastatic prostate cancer [27].

Cadherins are a group of adhesion molecules that have opposing roles in metastasis [28]. *E*-cadherin is a metastasis suppressor, while *N*-cadherin and cadherin-11 are metastasis promoters [28]. When tumor cells express high levels of *E*-cadherin, they have low levels of cadherin-11 and vice versa [28]. When *E*-cadherin was overexpressed in the highly metastatic breast cancer cell line MDA-MB-231, osteolytic lesions in a mouse model were decreased [29]. The opposite was found for cadherin-11 and *N*-cadherin. *N*-cadherin expression promotes cell migration and metastasis [30]. Cadherin-11 expression in MDA-MB-231 cells promotes bone metastasis [28]. Drugs targeting specific cadherins have not been reported.

## 6 Extravasation into the bone: Matrix metalloproteinases (MMPs)

In order to invade, cancer cells need to degrade their surrounding matrix both to leave the primary tumor and to enter the target organ [5]. In order to metastasize to bone, breast cancer cells need to degrade the hard, mineralized matrix of the bone [28]. Matrix metalloproteinases (MMPs) are a large family of membrane-bound and secreted zinc-dependent proteinases [2]. High levels of MMPs have been found in breast cancers and are associated with a poor prognosis [31, 32]. MDA-MB-231 cells express abundant MMP-1 [11], which is necessary for the initiation of osteoclastic bone resorption [33]. Therefore, breast cancer-produced MMPs, in particular MMP-1, may promote degradation of the bone matrix, allowing the breast cancer cells to enter the bone and promote osteolysis. However, MMP inhibitors failed initial clinical trials in cancer patients [34, 35], perhaps due to redundancy within the large MMP family [2].

## 7 Tumor-secreted factors: The creation of a vicious cycle within the bone microenvironment

Once within bone, a vicious cycle develops between breast cancer cells and the cells within the bone microenvironment (Fig. 1) [36]. Breast cancer cells secrete various factors that stimulate osteoblasts and osteoclasts and other cells within the bone; these in turn secrete factors that stimulate the tumor cells, creating a vicious cycle that renders bone metastases incurable [36]. The rest of this review focuses on the vicious cycle in bone. Interactions within the vicious cycle offer many targets for specific adjuvant treatments of bone metastasis.

Tumor-secreted factors play critical roles in breast cancer bone metastasis [36]. The tumor-secreted factors can be divided into two categories: bone-resorbing factors (indirect and direct osteoclast-stimulators) and bone-forming factors (osteoblast-stimulators) [36]. Cancer cells typically secrete multiple factors from both categories [36]. Some tumor-secreted bone-resorbing factors include: parathyroid hormone-related protein (PTHrP), interleukin-6 (IL-6), interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), and interleukin-11 (IL-11) [2]. Of these, PTHrP is the most studied and has been shown to play a central role in promoting osteolytic metastases, following its initial identification as the causal factor in humoral hypercalcemia of malignancy [4]. PTHrP acts through a shared receptor with parathyroid hormone (PTH) [37], the PTH/PTHrP receptor, but produces an opposite effect on bone formation than PTH. PTHrP secretion induces bone loss, while intermittent PTH secretion activates bone formation [38, 39]. PTHrP indirectly increases osteoclastic bone resorption by stimulating receptor activator of NFkappaB ligand (RANKL) expression on osteoblasts and bone stromal cells [40]. Mouse models of bone metastasis have established a causal role of PTHrP in breast cancer bone metastasis [39]. Inhibiting PTHrP with neutralizing antibodies reduced osteolytic lesions produced by MDA-MB-231 cells in mouse models [39]. A neutralizing antibody against PTHrP is currently in clinical trials to treat breast cancer bone metastasis. Even though PTHrP plays a significant role in osteolytic bone metastases, PTHrP expression in the primary tumor is not correlated with the presence of bone metastases in breast cancer patients and is associated with a better prognosis [41]. Therefore, PTHrP effects on bone metastases are local. Interleukins 6 and 11 and VEGF also increase osteoclast formation and activity via the RANK ligand pathway, while IL-8 acts directly and indirectly on osteoclasts [42–44].

A tumor-secreted peptide with a major causal role in osteoblastic metastasis is endothelin-1 (ET-1). Selective inhibition of the endothelin A (ETA) receptor decreased osteoblastic metastases due to ET-1-secreting ZR-75-1 breast cancer cells in a mouse model [45]. An ETA receptor antagonist is currently in clinical trials in men with advanced prostate cancer. Additional bone-secreted factors may be identified as targets for adjuvant treatment of bone metastases. Other potential tumor-secreted bone-forming factors include: adrenomedullin (AM), PTHrP fragments, cysteine-rich protein 61 (Cyr61), and connective tissue growth factor (CTGF) which are reviewed in Chirgwin,

Mohammad, & Guise (2004) and Clines & Guise (2005) [4, 46]. Many of the known tumor-secreted factors, both osteolytic and osteoblastic, are regulated by the hypoxia-induced Hif1α pathway and the TGFβ signaling pathway [11, 47, 48]. These two pathways are active in the bone microenvironment and are important targets for treatment of bone metastases.

## 8 Bone-seeking breast cancer clones: A tool to define important bone metastatic factors

Cancer cell clones that reproducibly metastasize to specific sites within the body have been selected by serial passaging in mice. These site-selective clones have revealed important characteristics involved in bone-specific metastasis. Kang et al. (2003) defined a bone metastatic gene profile by comparing MDA-MB-231 breast cancer cells that preferentially spread to the bone or the adrenal medulla [11]. The bone metastatic gene profile was independent of a poor prognosis gene profile identified by van't Veer et al. (2002) [49]. Cells with these gene profiles existed as a subpopulation of the parental cells [11]. The genes within the bone metastatic profile included genes involved in homing to bone, angiogenesis, invasion, and osteoclast recruitment [11]. A total of 43 genes were identified among which IL-11, CTGF, CXCR4, and MMP-1 were the most overexpressed in the bone metastatic clones [11]. Only combined expression of these genes significantly enhanced bone metastasis formation by poorly metastatic MDA-MB-231 cells [11]. This study demonstrates that a combination of factors contributes to breast cancer bone metastasis. Therefore, future treatment strategies should target multiple steps in the metastatic cascade.

Yoneda et al. (2001) also characterized a bone-seeking MDA-MB-231 clone (MDA-231BO) compared to a brain-seeking clone and the parental population [50]. The bone-seeking clone expressed higher levels of PTHrP, exhibited an increased growth-stimulatory response to IGF-1, and had altered responses to TGFβ compared to the parental cells [50]. Further studies by Myoui et al. (2003) found higher levels of tyrosine kinase c-Src activity in the bone-seeking clones [51]. Expression of a constitutively active c-Src in MDA-MB-231 cells increased bone metastases while, a c-Src inhibitor and expression of a kinase-dead cSrc decreased skeletal metastases [51, 52]. C-Src activity is essential for osteoclastic bone resorption[52], so c-Src inhibitors may be particularly useful against osteolytic bone metastases [53].

## 9 The bone soil

The remainder of this review focuses on characteristics of the bone 'soil' that make it a hospitable place for the breast cancer 'seed' to grow. Bone is composed of a hard, mineralized bone matrix that is constantly being remodeled [4]. The two main bone cells are the osteoclast (bone-resorbing cell) and the osteoblast (bone-forming cell) [4]. Osteoclasts are multinucleated cells derived from monocyte/macrophage precursors [4, 54]. The formation of osteoclasts requires the osteoblast [4]. Osteoblasts and stromal cells produce macrophage-colony stimulating factor (M-CSF), which stimulates precursor cells of the macrophage lineage to express the receptor RANK [4]. Osteoblasts and stromal cells then express receptor activator of NFkappaB ligand (RANKL), which binds to RANK to stimulate osteoclast differentiation, activation, and survival [4]. Osteoclast activation is opposed by the secreted RANKL-binding protein, osteoprotegerin, also produced by osteoblasts [4]. Balanced remodeling of the skeleton occurs due to the coupled actions of the osteoclast and the osteoblast [54]. The osteoclasts resorb bone leaving a pit in which the osteoblasts (bone-forming cells) can form new bone [54]. Tumor cells can unbalance coupling in the bone microenvironment resulting in net bone formation or bone loss [4].

## 10 The bone matrix: A rich source of growth factors

The bone matrix is a rich source of immobilized growth factors that can be released during bone resorption [55]. These growth factors drive a vicious cycle that contributes to the incurability once breast cancer cells enter bone [36]. Breast cancer cells stimulate osteoclastic bone resorption, which releases the immobilized growth factors from the matrix [36]. These growth factors act back on the tumor cells and the other cells within the bone to potentiate the vicious cycle [36]. These bone matrix growth factors therefore fertilize the bone soil for the breast cancer seed.

Hauschka et al. in 1986 identified a list of growth factors within the bone matrix [55]. In descending order of abundance within the bone matrix, insulin-like growth factor II (IGFII), insulin-like growth factor I (IGFI), transforming growth factor β (TGFβ), bone morphogenetic proteins (BMPs), fibroblast growth factors (FGF1 & 2), and platelet derived growth factor (PDGF) were isolated from the bone matrix based on their ability to bind heparin and identified using immunoassays available at the time [55]. Whether most of these proteins are released in active form from bone *in vivo* has not been tested. The acid secreted during osteoclastic bone resorption could inactivate some of the bone matrix growth factors. Identifying what growth factors are active after bone resorption will allow further understanding of what factors contribute to the vicious cycle of bone metastasis.

The list of growth factors in the bone matrix is 20 years old and needs to be revisited. It is known that osteoblasts secrete other factors that are not on the current list of bone-growth factors. For example, the six CCN proteins (cysteine-rich protein 61 (CYR61), connective tissue growth factor (CTGF), nephroblastoma overexpressed (NOV), and wnt-induced secreted proteins 1, 2, and 3 (WISP1, 2 &3)) and osteoprotegerin are secreted by osteoblasts [4, 56, 57]. These potential heparin-binding proteins may be additional components of the bone matrix. Other osteoblast-secreted factors include: adrenomedullin (AM), tissue inhibitors of metalloproteinase (TIMPs), vascular endothelial growth factor (VEGF), colony stimulating factor-1 (CSF-1), interleukin-6 (IL-6), stanniocalcins, and hepatocyte growth factor (HGF) [58–65]. These non-heparin binding factors could also be incorporated into the bone matrix.

## 11 TGFβ (transforming growth factor β)

TGFβ is the third most abundant growth factor in the bone matrix [55]. TGFβ is released from the bone matrix and activated by osteoclastic resorption [66]. TGFβ stimulates breast cancer cells to secrete factors such as CTGF, IL-11, and PTHrP that drive bone metastases [11, 48]. The role of TGFβ in breast cancer changes during tumor progression [67]. During the early stages of carcinogenesis, TGFβ inhibits tumor growth, but in advanced cancers, growth inhibition is lost, while TGFβ continues to stimulate breast cancer expression of pro-metastatic factors [67]. Expression of a dominant negative TGFβ receptor in MDA-MB-231 breast cancer cells blocked responsiveness to TGFβ and decreased bone metastases in mice [48]. TGFβ increased breast cancer secretion of PTHrP by a Smad-dependent and a Smad-independent (MAPK) pathway [68]. These pathways provide additional targets for treatment. Indeed, TGFβ inhibitors are effective in preclinical models to block metastases [69–72].

## 12 IGFs (insulin-like growth factors)

It is unknown whether IGFs are released in an active form by osteoclasts during bone resorption. IGF-II is the most abundant and IGF-I is the second most abundant growth factor within the bone matrix [55]. Inhibition of IGF signaling inhibited bone lesion formation by myeloma and prostate cancer cells in mouse models [73, 74]. Overexpression of IGF-1R in neuroblastoma cells increased osteolytic lesions formed by intratibial injection of neuroblastoma cells in mice [75]. However, overexpression of IGFI in prostate cancer cells did not increase bone lesions in a similar model [76]. Further research may show a role of bone matrix IGFs in breast cancer bone metastasis.

## 13 Bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), and platelet-derived growth factors (PDGFs)

Less abundant growth factors found within the bone matrix include the BMPs, FGF-1 and -2, and PDGFs [55]. BMPs are a family of growth factors that stimulate bone and cartilage formation [77]. Breast cancer cells express BMPs and BMP receptors [78]. BMPs have both growth inhibitory and stimulatory effects on cancer cells [79]. BMP-2 treatment inhibited proliferation of MDA-MB-231 and MCF-7 breast cancer cells *in vitro* [78–80]. However, overexpression of a dominant negative type II bone morphogenetic protein receptor in T47D breast cancer cells inhibited proliferation, indicating that BMPs that act through the BMPRII may stimulate proliferation of breast cancer cells [79]. Therefore, different BMPs may have different growth effects on breast cancer cells [79]. BMPs may also play a role in breast cancer progression. Increased expression of the bone morphogenetic protein receptor IB is associated with increased tumor grade, proliferation, cytogenetic instability, and poor prognosis of estrogen receptor-positive breast carcinomas [81]. Overexpression of BMP-2 in MCF-7 breast cancer cells increased the invasive ability of these cells *in vitro* and enhanced estrogen-independent growth of these cells in a xenograft mouse model [82].

FGFs are a family of 23 growth factors that signal through tyrosine kinase receptors (FGFR1-4) and are involved in bone growth and development [83, 84]. Mutations in FGF receptors have been associated with many skeletal disorders including achondroplasia (a form of skeletal dwarfism), Apert syndrome, Beare–Stevenson cutis gyrata, Crouzon syndrome, Pfeiffer syndrome, non-syndromic craniosynostosis, osteoglophonic dysplasia, and Muenke syndrome [84]. FGFs regulate chondrocyte and osteoblast proliferation and osteoclast differentiation [84–86]. FGFs are also involved in tumor growth and angiogenesis [87]. Breast cancers express both FGFs and FGFRs [87]. Breast tumors have higher expression of FGFR-1 than normal breast epithelium [87]. Overexpression of FGF-1 and FGF-4 in MCF-7 breast cancer cells increased tumor growth, blood flow rate, and lung metastases *in vivo* [88]. FGFs may also affect the invasive ability of breast cancer cells by stimulating secretion of matrix metalloproteinases. FGF-2 treatment of MCF-7 cells stimulated MMP-9 secretion *in vitro* [89].

PDGFs are multifunctional cytokines that stimulate both osteoclasts and osteoblasts [90]. PDGF stimulates bone resorption indirectly by inducing osteoblastic secretion of interleukin-6 (IL-6) or by direct actions on the osteoclast [91]. Breast cancer cells secrete PDGFs [90]. High PDGF plasma and tumor tissue levels are associated with a poorer prognosis for breast cancer, including increased metastases,

lower chemotherapeutic response, and lower survival [92, 93]. Tumor-secreted PDGF-BB may play a role in osteoblastic metastases formed by breast cancers [90]. Reduction of PDGF-BB in MCF-7 breast cancer cells overexpressing the oncogene Neu decreased osteoblastic bone metastases in nude mice [90]. Overexpression of PDGF-BB in MDA-MB-231 breast cancer cells, which normally produce osteolytic lesions, produced osteoblastic lesions [90]. The PDGF receptor is also found on breast tumor cells and endothelial cells [94]. A PDGF receptor tyrosine kinase inhibitor decreased growth of breast cancer cells injected into the tibia of mice [94].

Bone-matrix derived BMPs, FGFs, and PDGFs may all play a role in bone metastasis. As discussed above, they all have identified roles in bone formation and cancer. However, it is currently unknown whether or not these factors are released in an active form and in a high enough concentration from the bone matrix to play a significant role in bone metastases. Additional research is needed to establish a role of these less abundant growth factors in breast cancer bone metastases.

## 14 The bone matrix: A rich source of calcium

In addition to growth factors, the bone is a rich source of calcium [28]. The bone matrix consists largely of hydroxyapatite (calcium phosphate) mineral crystals [28]. During bone remodeling, free calcium reaches locally high concentrations between 8–40 mM [95]. Breast cancer cells express calcium-sensing receptors, CaSR [96]. Activation of CaSR by free calcium increases PTHrP expression by breast cancer cells [96]. Therefore, bone-stored calcium may contribute to the increased levels of PTHrP found in breast cancer cells that have metastasized to the bone compared to the primary tumor and other sites of metastases [41, 97–99]. Calcium may therefore be an important component within the bone microenvironment contributing to breast cancer bone metastasis. The calcium-sensing receptor may be a useful target to inhibit breast cancer bone metastasis, since small molecule drugs that act on the calcium-sensing receptor have been developed [100].

## 15 Other cells within the bone microenvironment

Current research has mostly focused on the interactions between the tumor cells and the bone cells within the bone microenvironment. However, a large number of additional cells within the bone microenvironment may also contribute to breast cancer bone metastases. They can be broken up into four groups: stem cells that give rise to both hematopoietic and mesenchymal cells, hematopoietic cells (immune cells, platelets, and erythrocytes), mesenchymal cells (adipocytes, fibroblasts and other stromal cells, chondrocytes, and smooth muscle cells), and endothelial cells [54]. These cells may interact with the tumor cells and contribute to the vicious cycle of breast cancer bone metastasis. These additional cells make the vicious cycle model a more complete but also a more complex model (Fig. 1). Understanding of all the interactions within the bone microenvironment may reveal additional targets for the treatment of bone metastases.

## 16 Stem cells

Stem cells are undifferentiated cells that are capable of differentiating into a variety of cell types [101]. They are capable of self-renewal and often remain quiescent [101]. Hematopoietic and mesenchymal stem cells are both found within the bone microenvironment [101, 102]. Hematopoietic stem cells differentiate into all of the immune cells, platelets, erythrocytes, and osteoclasts in bone [54]. They have been observed along endosteal surfaces of bone and interact with osteoblasts [103]. Constitutive activation of the PTH/PTHrP receptor on osteoblasts increased hematopoietic stem cell number in mice [104]. PTH injection also increased stem cell number in wildtype mice [104]. Therefore, osteoblasts can regulate hematopoietic stem cells through activation of the PTH/PTHrP receptor [104]. Increased activation of the osteoblastic PTH/PTHrP receptor in bone metastases may also increase hematopoietic stem cell formation in bone metastases. Hematopoietic stem cells also influence osteoblastic secretion of IL-6 and MIP-1$\alpha$ [103]. Bone marrow mesenchymal stem cells give rise to osteoblasts as well as fibroblasts and adipocytes in bone. They have recently been implicated in neuroblastoma bone metastasis [102]. Instead of acting directly on the bone cells, neuroblastoma cells secrete a factor (suggested by the authors to be epidermal growth factor (EGF)) that causes bone marrow mesenchymal stem cells to secrete IL-6, which in turn activates osteoclasts and bone destruction [102]. The data suggest a role of bone marrow mesenchymal stem cells in the vicious cycle of bone metastasis. However, IL-6 has been extensively studied in bone and has not proven to be a major osteolytic factor [42]. Further research needs to be done to see if hematopoietic stem cells and bone marrow mesenchymal stem cells play a role in the vicious cycle of breast cancer bone metastasis.

Tumor cells may also carry stem cells within their population [101, 105] that may play a role in the ability of cancer cells to remain dormant within the bone [101]. Breast cancer cells are often detected in bone early in patients but actual bone metastases are not detected until years later [101, 106, 107]. The bone microenvironment

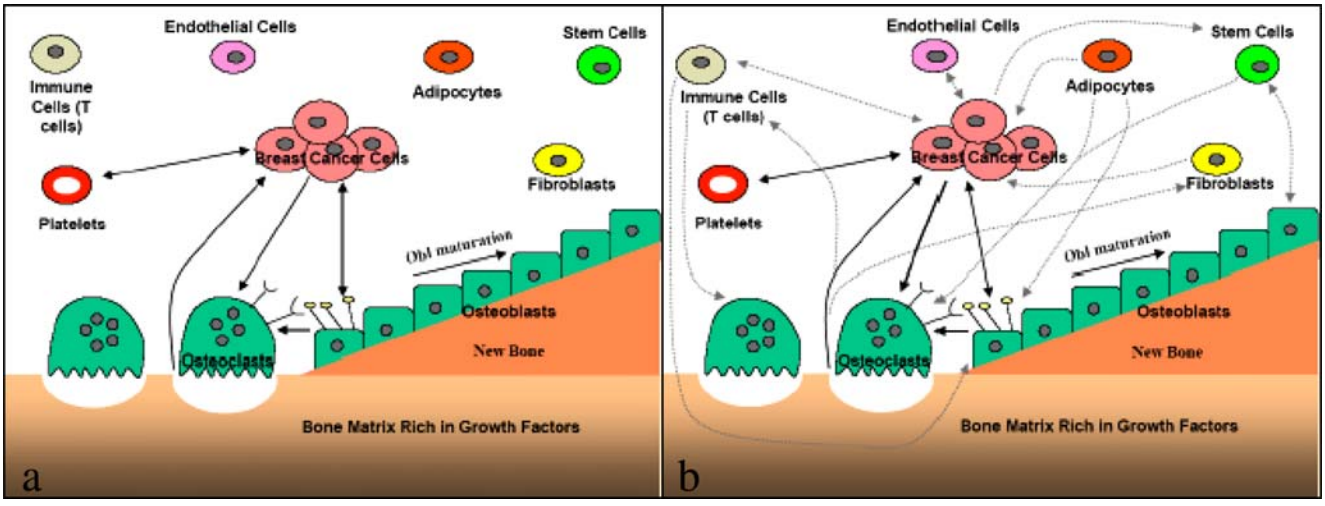


**Fig. 1** Adding to the 'vicious cycle' of breast cancer bone metastasis: Within bone, breast cancer cells interact with bone cells and the other cells within the bone, creating a vicious cycle that makes bone metastases incurable. Breast cancer cells secrete factors that stimulate the other cells within bone to secrete factors that act back onto the breast cancer cells. The bone contains hematopoietic cells (immune cells, platelets, and osteoclasts), endothelial cells, mesenchymal cells (adipocytes, fibroblasts, and osteoblasts), and stem cells that give rise to both hematopoietic and mesenchymal cells. Panel (a) demonstrates the experimentally established interactions within the vicious cycle. Breast cancer cells secrete factors that both directly and indirectly stimulate osteoclastic bone resorption. Bone resorption releases growth factors that stimulate breast cancer cells. Breast cancer cells can also secrete factors that stimulate osteoblasts. Panel (b) shows the potential interactions that may also occur within the bone. Adding the roles of non-bone cells found within the bone microenvironment such as endothelial cells, stem cells, T cells, platelets, adipocytes, and fibroblasts will produce a more complete and complex model of breast cancer bone metastasis. *Solid black lines* indicate established interactions within the vicious cycle. *Dotted gray lines* indicate potential additional interactions that may also occur within the vicious cycle

may provide niches in which tumor stem cells can remain dormant until eventual activation of tumor growth and metastasis formation [101]. Since stem cells or quiescent cells are often resistant to chemotherapeutic treatment, maintenance of breast cancer stem cells within the bone may contribute to the incurability of the disease [101]. Most of the work described in this review has been done using mouse models injected with single cell clones produced by antibiotic selection from a parental population of cancer cells. The clonal selection eliminates tumor stem cells from the model, but the clones can still produce tumors, suggesting that tumor stem cells may not be necessary for bone metastases in mouse models.

## 17 Hematopoietic cells: T cells and platelets

Hematopoietic cells found within the bone include the erythrocytes, immune cells, osteoclasts, and platelets [54]. T cells are immune cells found within the bone that may play a role in the vicious cycle of breast cancer bone metastasis (reviewed by Fournier et al., 2006) [108]. They are derived from hematopoietic stem cells in the bone marrow and are activated within the thymus [108]. T cells can express RANK ligand and increase bone resorption [109]. They express membrane proteins that allow them to interact with osteoblasts, causing secretion of IL-6 [110]. Peripheral T cells from breast cancer patients secrete TNFα, which can promote osteoclastogenesis [111]. Tumor-secreted factors such as PTHrP, IL-7, or IL-8 may activate T cells, adding another component to the vicious cycle [108]. In addition, the bone matrix may also influence the immune function of T cells in bone [108]. The release of TGFβ during bone resorption may suppress the immune functions of T cells, preventing the cytotoxic action of T cells against the tumor cells [108]. TGFβ inhibits T-cell proliferation, natural killer cell function, and antigen presentation [108, 112]. Therefore, the increased bone resorption due to tumor-secreted factors and T cell-secreted factors may enhance survival of the tumor cells within the bone by releasing TGFβ from the bone matrix, and causing inhibition of the immune response of T cells to tumor cells [108]. Current models of bone metastasis rely on immunocompromised (Nude or SCID) mice [108]. Nude mice lack T cells, while SCID mice lack both B and T cells [113, 114]. Models that incorporate immune cells (T cells) may serve as more complete tools to study breast cancer bone metastasis [108].

Platelets also arise from hematopoietic stem cells and are involved in blood clotting [54, 115]. When breast cancer cells travel through the blood stream, platelets may aid metastasis by coating the cancer cells, protecting them from immune cells within the blood and helping them attach to endothelial cell walls [116]. MDA-MB-231 breast cancer cells induce platelet aggregation and secretion of LPA [117].

Overexpressing the LPA1 receptor in bone-specific MDA-MB-231 cells (MDA-BO2) increased tumor growth and bone metastases [117]. The platelet antagonist integrellin inhibited bone metastases produced by both the LPA1-overexpressing and parental MDA-BO2 breast cancer cells [117]. In addition, siRNA knockdowns of LPA1 and LPA1 receptor antagonists significantly reduced bone metastases produced by MDA-BO2 [118]. The experiments suggest a causal role of platelet-secreted LPA in promoting breast cancer bone metastasis. Within the vicious cycle, breast cancer cells stimulate platelets to release factors (LPA) that act back onto the breast cancer cells, increasing bone metastasis formation. Inhibiting LPA binding to LPA1 may be a useful target for breast cancer bone metastasis treatment.

## 18 Mesenchymal cells: Adipocytes and fibroblasts

Mesenchymal cells are the most abundant group of cells in bone; they include adipocytes, fibroblasts and related stromal cells, chondrocytes, osteoblasts, and osteocytes [54]. Adipocytes (fat cells) are energy-storage cells that secrete various factors including hormones, growth factors, and adipocytokines such as leptin, adiponectin, TNFα, IL-6, heparin-binding epidermal growth factor, IGFII, and adipsin [119]. Patients with excessive adipose tissue have an increased risk of developing breast, colorectal, endometrial, renal, and esophageal cancers [120]. Elliot et al. (1992) demonstrated that the addition of adipose tissue enhanced growth and metastasis of the murine mammary carcinoma cell line SP1 in mice [121]. Only when adipose tissue was injected along with SP1 cells were they able to form tumors and metastases [121]. Manabe et al. (2003) showed that mature adipocytes promoted the growth of estrogen receptor-positive breast cancer cells *in vitro* [119]. Iyengar et al. (2003) showed that adipocyte-secreted factors increased breast cancer cell proliferation, motility, migration, and angiogenesis [122]. In particular, the adipocyte-secreted factor leptin stimulates growth of estrogen receptor-positive breast cancer cells, which express leptin receptors [123]. Adipocyte-secreted factors induce the transcription of several genes involved in breast cancer proliferation, invasiveness, survival, angiogenesis, and stabilization of proto-oncogenes [122].

The role of adipocytes within the breast cancer-bone microenvironment has not been well studied, but adipocytes in the bone may promote tumor growth and regulate osteoblast proliferation. Mature adipocytes secrete factors that inhibit osteoblast proliferation *in vitro* [124]. Adipocyte-secreted factors such as leptin, TNFα, and IL-6 can affect bone formation [124]. Leptin enhanced osteoblastic differentiation of a human marrow stromal cell line [125]. TNFα and IL-6 stimulate bone resorption [124]. Further study is needed to assess the contributions of adipocytes to breast cancer bone metastasis.

Fibroblasts secrete pro-metastatic factors such as hepatocyte growth factor (HGF) [126]. Fibroblasts secrete HGF as an inactive precursor that must be processed for activation [126]. Inhibiting HGF activation decreased fibroblast-induced breast cancer cell invasion [126]. Preventing breast cancer cell inactivation of HGF increased migration, proliferation, and invasion of MDA-MB-231 breast cancer cells [126]. MDA-MB-231 breast cancer cells stimulated expression of the cell surface heparan sulfate proteoglycan, syndecan-1 in co-cultured fibroblasts [127]. Syndecan-1 expression in fibroblasts increased breast cancer cell proliferation *in vitro* [127] and increased breast cancer tumor growth and angiogenesis *in vivo* [128]. Therefore, fibroblast–breast cancer cell contact can promote breast cancer tumor growth and angiogenesis.

Inactive MMP2 is released from fibroblasts by the interaction of fibronectin on breast cancer cell surfaces with the cell surfaces of fibroblasts [129]. The released, but inactive MMP2 can than be activated by breast cancer cells, increasing their invasiveness [129]. The secretion of Cyr61 by breast cancer cells stimulates autocrine migration by enhancing MMP1 secretion from fibroblasts [130]. Thus, fibroblast–breast cancer cell interactions can enhance the invasiveness of breast cancer cells.

Fibroblasts in bone may also respond to bone-derived growth factors. The bone-derived growth factor TGFβ increases fibroblast expression of stromelysin-3, a metalloproteinase that may enhance metastasis [131]. Fibroblasts may also stimulate tumor-associated macrophage differentiation into osteoclasts [132]. Fibroblasts isolated from metastatic melanomas can activate osteoclasts in a RANKL-dependent manner [132]. Therefore, fibroblasts may be additional contributors to the vicious cycle.

## 19 Endothelial cells

The stimulation of new blood vessel formation by endothelial cells is essential for cancer cell survival [133]. Higher bone marrow microvessel density is associated with bone metastasis in patients with breast cancer [133]. Many bone-active, tumor-secreted factors (e.g., vascular endothelial growth factor (VEGF), cysteine-rich protein 61 (Cyr61), and adrenomedullin (AM)) and bone matrix growth factors (e.g., fibroblast growth factors (FGFs) and platelet-derived growth factors (PDGFs)) also stimulate endothelial cell proliferation, differentiation, and angiogenesis [87, 94, 133–136]. Overexpression of the actin-binding protein cortactin in breast cancer cells increased bone metastasis formation in an intracardiac injection model by increasing transendothelial invasion and tumor-endothelial

adhesion [137]. Proteins like cortactin, when overexpressed in breast cancer cells, may increase tumor-endothelial cell interactions within the bone microenvironment and enhance breast cancer bone metastases.

## 20 Conclusions

- A large number of patients with advanced breast cancer develop bone metastases.
- Bone metastases are painful and currently incurable, leaving the need for the identification of new treatment targets.
- Breast cancer cells preferentially form metastases within the bone. Characteristics of both the breast cancer cell ("the seed") and the bone ("the soil") contribute to the preferential spread of breast cancer cells to bone.
- Breast cancer cells express genes that allow them to preferentially spread to bone. They express the chemokine receptor CXCR4, the integrin receptor alphav-beta3, cadherins, and bone-resorbing and bone-forming factors that contribute to bone metastasis formation.
- The bone is a fertile soil in which the bone matrix provides immobilized growth factors that can be released by osteoclastic bone resorption. New research needs to be done to potentially identify additional factors within the bone.
- Within the bone, breast cancer cells interact with bone cells and other cells within the bone, creating a vicious cycle that renders bone metastases incurable. Breast cancer cells secrete factors that stimulate other cells within the bone to secrete other factors that can act back on the breast cancer cells.
- Many cells including stem cells, T cells, platelets, adipocytes, fibroblasts, and endothelial cells, in addition to the bone cells are found within the bone microenvironment and may play a role in breast cancer bone metastasis.
- Current models of bone metastasis exclude immune cells. Additional bone metastasis models need to be developed to study the complete interactions within the bone microenvironment.
- There are many complex interactions within the breast cancer bone microenvironment. Understanding these interactions may allow for the development of new treatments that cure this deadly disease.
- Targeting multiple areas of bone metastasis may be a more complete approach to treating breast cancer bone metastases.

## 21 Key unanswered questions

- Are there additional factors immobilized within the bone matrix besides the list of growth factors from Hauschka produced in 1986?
- What are the roles of adipocytes, fibroblasts, immune cells, platelets, stem cells, and endothelial cells within the breast cancer bone microenvironment? Do these additional cells significantly contribute to breast cancer bone metastasis? What models of bone metastasis can be used that incorporate all of these factors?
- How can all the complex interactions in the bone microenvironment be blocked to treat breast cancer bone metastasis?

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**Supporting Data:**

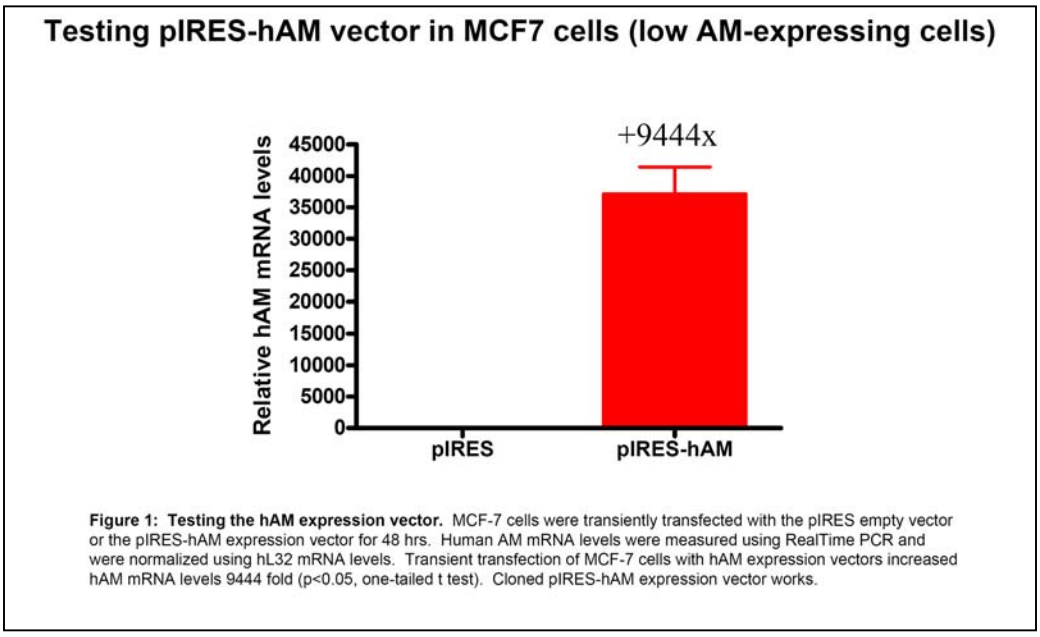


**Figure 1: Testing the hAM expression vector.** MCF-7 cells were transiently transfected with the pIRES empty vector or the pIRES-hAM expression vector for 48 hrs. Human AM mRNA levels were measured using RealTime PCR and were normalized using hL32 mRNA levels. Transient transfection of MCF-7 cells with hAM expression vectors increased hAM mRNA levels 9444 fold ($p<0.05$, one-tailed t test). Cloned pIRES-hAM expression vector works.

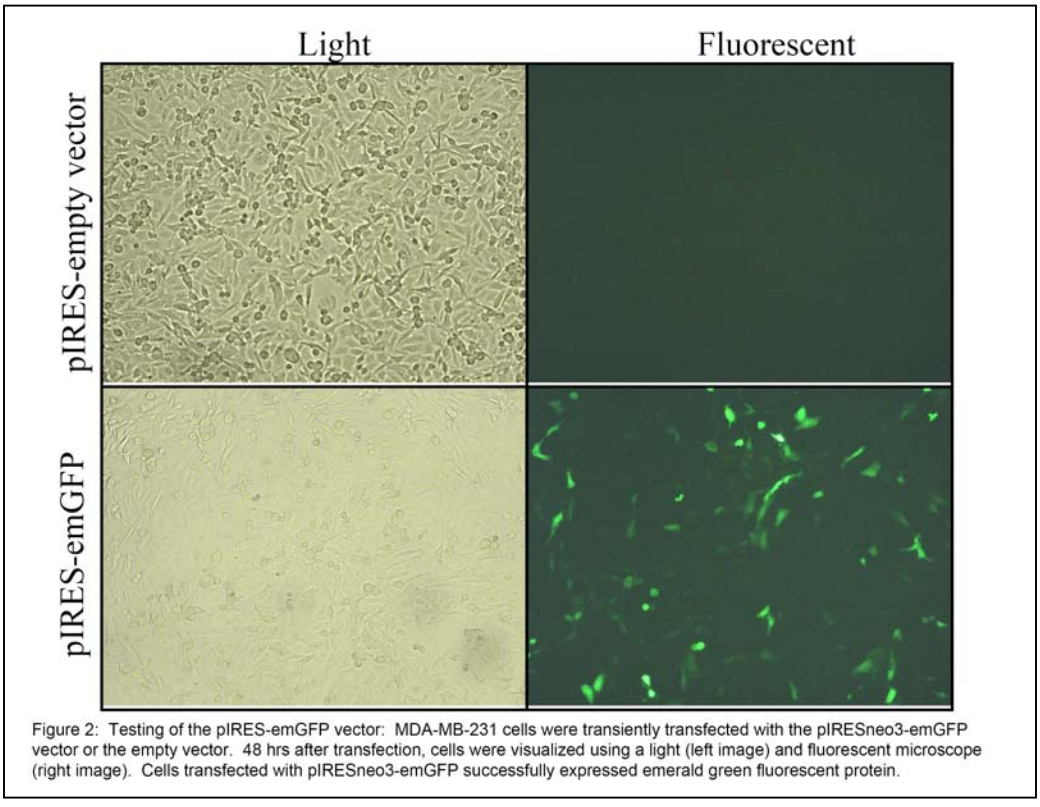


Figure 2: Testing of the pIRES-emGFP vector: MDA-MB-231 cells were transiently transfected with the pIRESneo3-emGFP vector or the empty vector. 48 hrs after transfection, cells were visualized using a light (left image) and fluorescent microscope (right image). Cells transfected with pIRESneo3-emGFP successfully expressed emerald green fluorescent protein.

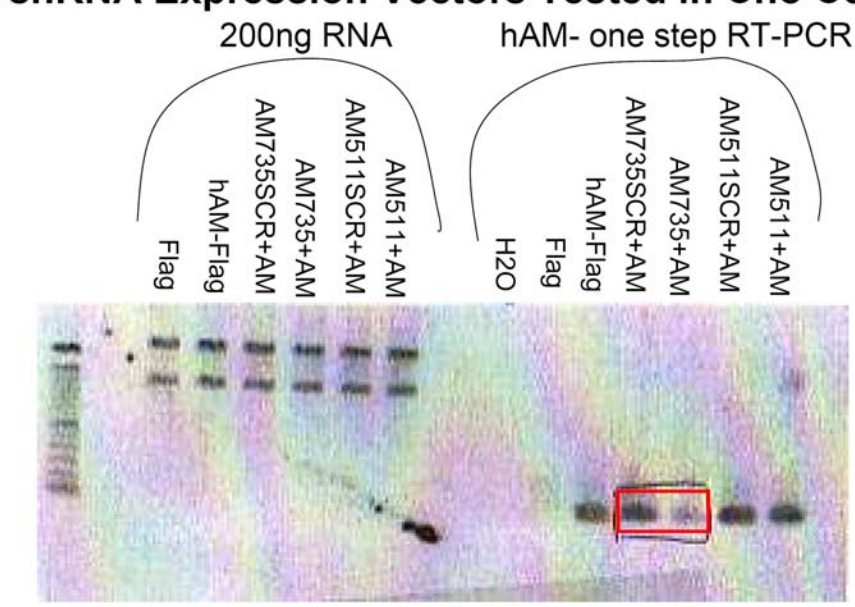


**Figure 3: Testing of shRNA expression vectors in CHO cells.** CHO cells were transiently co-transfected with hAM and AM- targeting shRNA expression vectors (AM735 and AM511) or control shRNA expression vectors (AM735SCR and AM511SCR) to test effectiveness of shRNA expression vectors. One step RT-PCR was performed using hAM primers and than PCR products were visualized on an agarose gel using ethidium bromide staining. Equal amounts of RNA were used in each PCR reaction for normalization. AM735 shRNA produced the largest decrease in hAM while the control version of this shRNA (AM735SCR) did not change the hAM level (boxed in red).

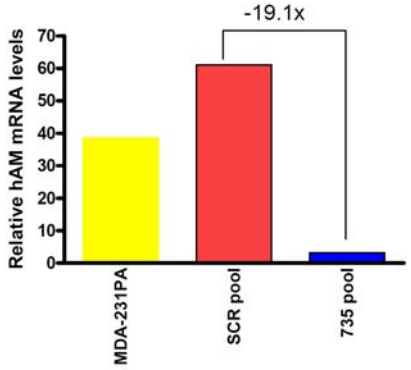


Figure 4: MDA-MB-231 AM knockdown stable pool: MDA-MB-231 cells were transfected with AM735 (AM-targeting shRNA) or AM735 SCR (negative control) shRNA. A stable pool was selected by treating the cells with 1100ug/mL G418. Relative hAM mRNA levels were compared using RealTime PCR. HL32 mRNA levels were used for normalization. The AM knockdown pool (735 pool) had a 19.1 fold decrease in AM compared to the negative control pool (SCR pool).

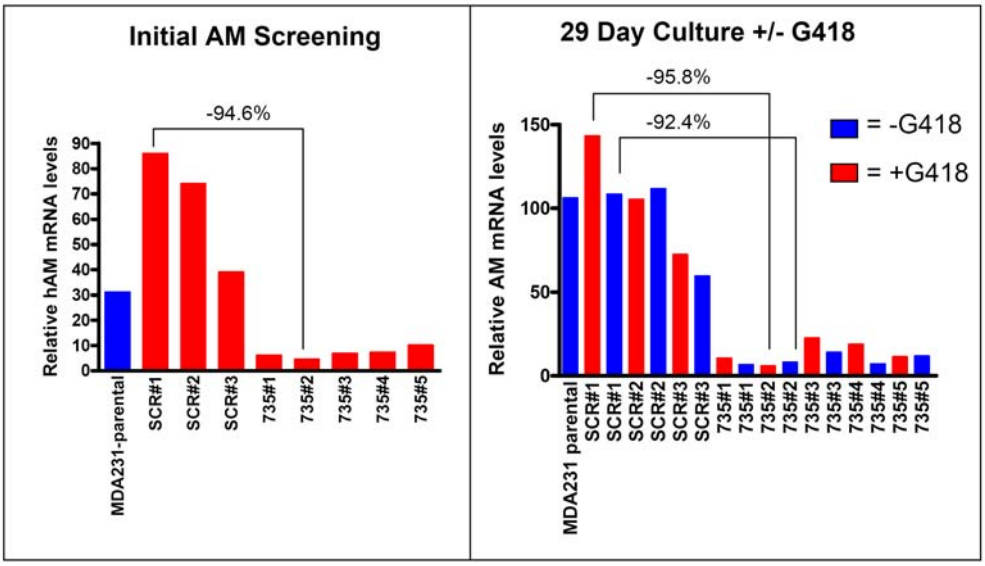


Figure 5: Testing of AM knockdown clones: Relative hAM mRNA levels of MDA-MB-231 stable clones were measured using RealTime PCR and were normalized using hL32 mRNA levels. Initial screening of relative hAM mRNA levels between knockdown clones (735#1-5) and control clones (SCR#1-3) showed a 94.6% knockdown between the highest control and the lowest knockdown (left figure). All the knockdown clones had lower AM mRNA levels compared to the control clones. To test the stability of the knockdown, the clones were cultured for 29 days with and without selection (+/- G418 (neomycin)). All the knockdown clones maintained a decreased level of AM mRNA after 29 day culture without G418 treatment (right figure).

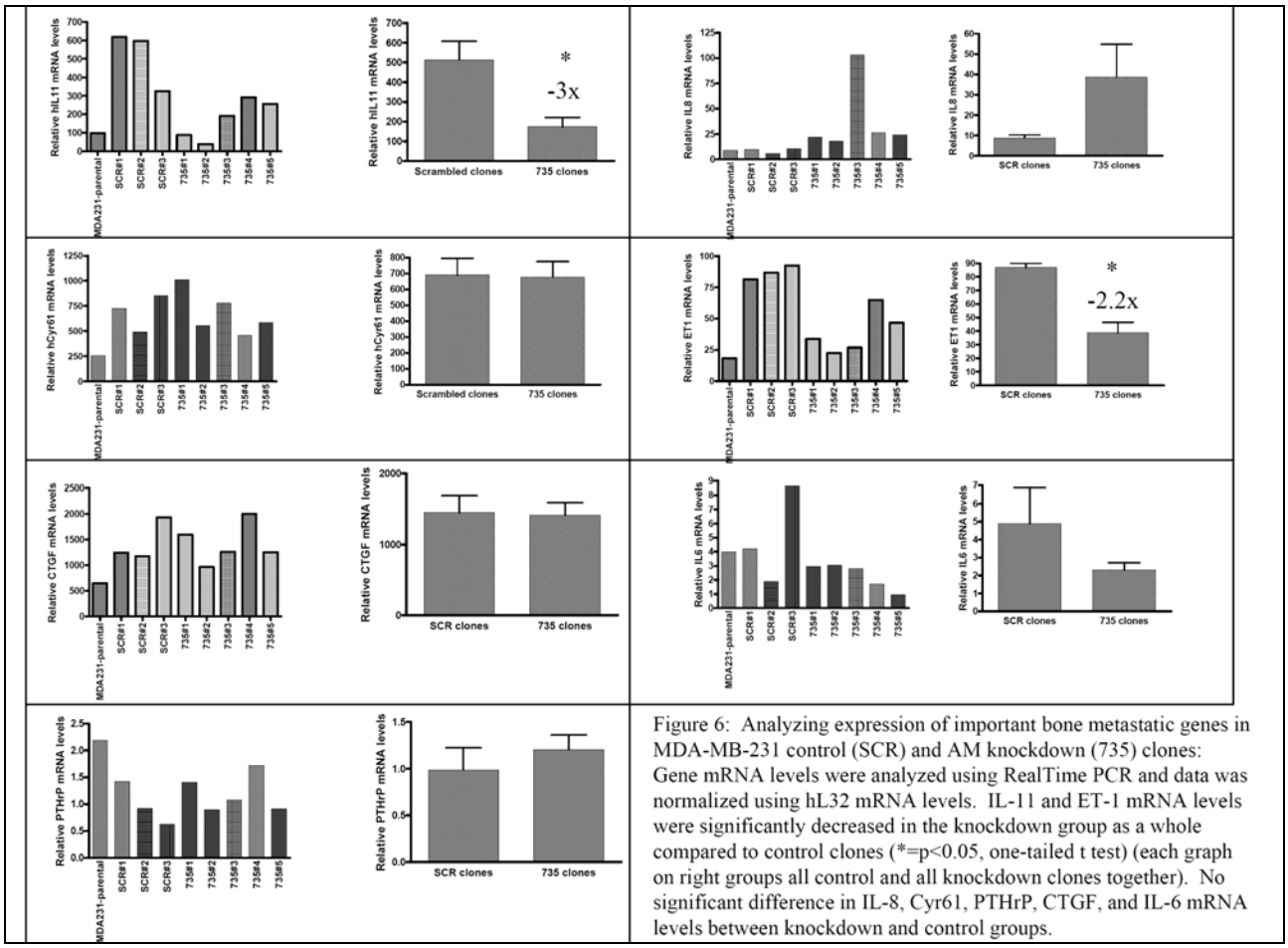


Figure 6: Analyzing expression of important bone metastatic genes in MDA-MB-231 control (SCR) and AM knockdown (735) clones: Gene mRNA levels were analyzed using RealTime PCR and data was normalized using hL32 mRNA levels. IL-11 and ET-1 mRNA levels were significantly decreased in the knockdown group as a whole compared to control clones (*=p<0.05, one-tailed t test) (each graph on right groups all control and all knockdown clones together). No significant difference in IL-8, Cyr61, PTHrP, CTGF, and IL-6 mRNA levels between knockdown and control groups.

## Confirmed Increase in RhoGDI2 and Decrease in AM in T47D COBRA2a Knockdown Cells

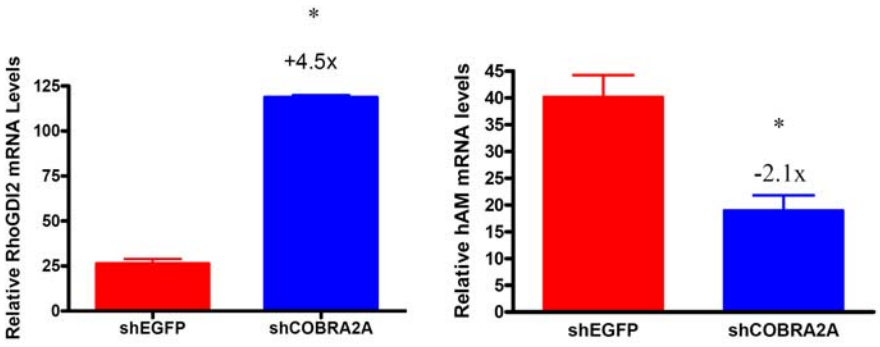


Figure 7: Confirmed increase in RhoGDI2 and decrease in AM in T47D COBRA knockdown cells. AM and RhoGDI2 mRNA levels of T47D shEGFP (negative control cells) and shCOBRA2A (COBRA knockdown cells) were compared using RealTime PCR. L32 mRNA levels were used for normalization. COBRA knockdown cells (shCOBRA2A) had increased RhoGDI2 and decreased AM compared to shEGFP (negative control) cells (*=p<0.05, one-tailed t test).

## Inhibiting ROCK (downstream effector of Rho) Does NOT Affect AM mRNA Levels in shEGFP T47D cells

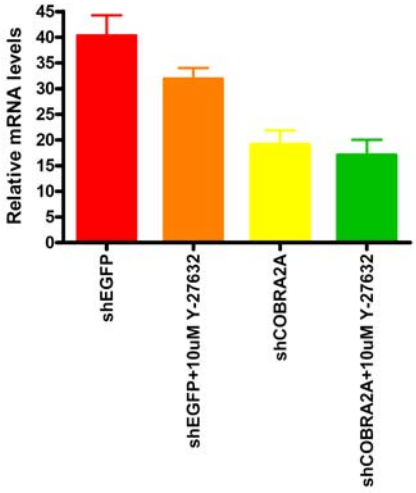


Figure 8: The effect of ROCK inhibition in T47D shEGFP cells: T47D shEGFP (negative control cells) and shCOBRA2a (COBRA knockdown cells) cells were treated for 24 hours with and without the ROCK inhibitor 10uM Y-27632. After 24 hour treatment, relative AM mRNA levels were determined using RealTime PCR and L32 mRNA levels for normalization. Inhibiting ROCK did not significantly change shEGFP's AM mRNA level ($p>0.05$, one-tailed t test).

## Overexpressing RhoGDI2 in MCF-7 Cells Did Not Cause a Significant Change in AM mRNA Levels

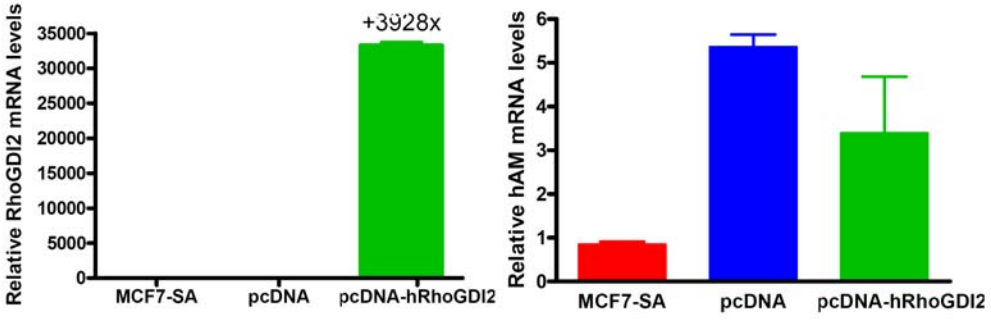


Figure 9: Overexpression of RhoGDI2 in MCF-7 cells does not change AM mRNA levels: MCF-7 cells were transiently transfected with the pcDNA empty vector or the pcDNA-hRhoGDI2 expression vector for 48 hours. Transfection increased RhoGDI2 levels 3928 fold ($p<0.05$, one-tailed t test) (left figure). Overexpression of RhoGDI2 did not change AM mRNA levels $p>0.05$, one-tailed t test) (right figure). mRNA levels were compared using RealTime PCR and L32 mRNA levels were used for normalization.

## Overexpressing RhoGDI2 in MDA-MB-231 Cells Did Not Cause a Significant Change in AM mRNA Levels or AM Promoter Activity

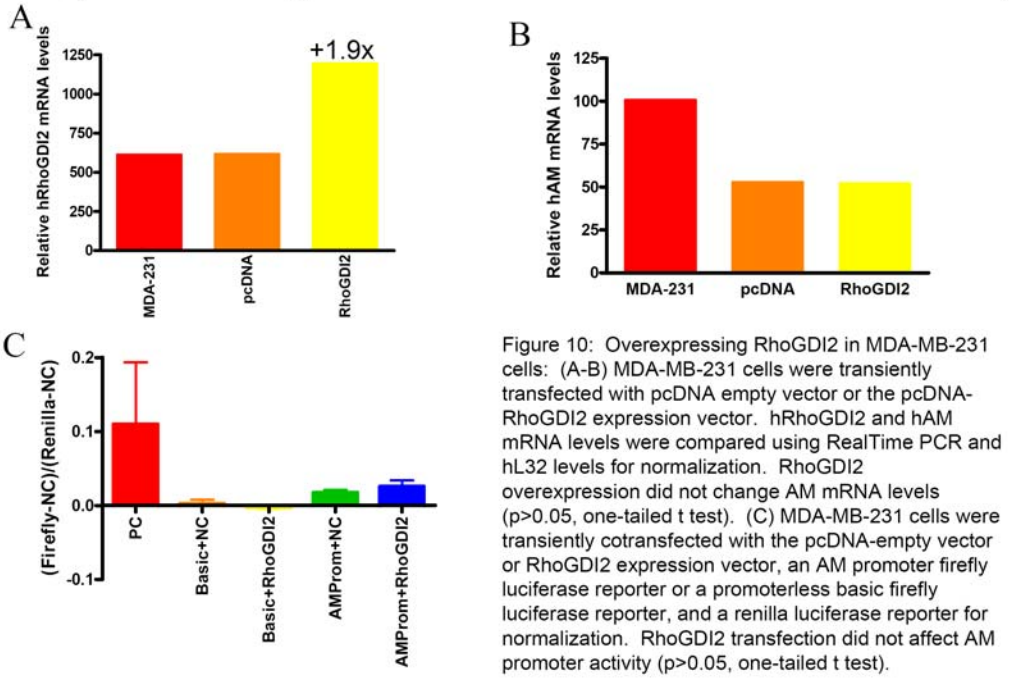


Figure 10: Overexpressing RhoGDI2 in MDA-MB-231 cells: (A-B) MDA-MB-231 cells were transiently transfected with pcDNA empty vector or the pcDNA-RhoGDI2 expression vector. hRhoGDI2 and hAM mRNA levels were compared using RealTime PCR and hL32 levels for normalization. RhoGDI2 overexpression did not change AM mRNA levels ($p>0.05$, one-tailed t test). (C) MDA-MB-231 cells were transiently cotransfected with the pcDNA-empty vector or RhoGDI2 expression vector, an AM promoter firefly luciferase reporter or a promoterless basic firefly luciferase reporter, and a renilla luciferase reporter for normalization. RhoGDI2 transfection did not affect AM promoter activity ($p>0.05$, one-tailed t test).